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Relevance of copper transporter 1 for cisplatin resistance in human ovarian carcinoma cells

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ABSTRACT

Defects in intracellular accumulation of the antitumour drug cisplatin are a commonly observed feature in the cells selected for cisplatin resistance. Copper transporter 1 (CTR1) has been suggested to play an important role in drug uptake and resistance. Here, we describe a detailed investigation of the involvement of CTR1 in cisplatin uptake and its relevance for cisplatin resistance using a well characterised sensitive/cisplatin-resistant cell line pair: A2780 human ovarian carcinoma cell line and its cisplatin-resistant variant A2780cis. A2780cis cells showed decreased cisplatin accumulation and lower CTR1 expression compared to A2780 cells. Co-incubation with copper sulphate affected neither cisplatin accumulation (determined by flameless atomic absorption spectrometry) nor its cytotoxicity (determined using an MTT-assay, MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide). In both cell lines, CTR1 was localised near the nucleus as found using confocal fluorescence microscopy. The steady-state localisation of the protein in perinuclear region appears to involve its continuous endocytosis from cell surface. In contrast to copper, cisplatin exposure had no influence on the sub cellular localisation of CTR1. Co-localisation between CTR1 and a fluorescent cisplatin analogue labelled with carboxyfluorescein-diacetate could be observed in vesicular structures when continuous retrieval of the protein from cell membrane was inhibited. Our results strongly suggest that CTR1 mediates cisplatin uptake in the cell lines studied. Upon its transport across the plasma membrane by CTR1 the platinum drug is likely to be internalised along with the protein. Our findings imply that reduced CTR1 expression accounts for decreased cisplatin accumulation and represents one of the determinants of cisplatin resistance in A2780cis cell line.

1. Introduction

Over the past decades, cisplatin remains a widely used antitumour drug applied for treatment of various solid tumours including testicular, ovarian, head and neck, and small cell lung cancers [1]. Development of resistance in the course of the chemotherapy is, however, common and often leads to treatment failure. In order to understand mechanisms of cisplatin resistance, multiple studies have focused on biochemical and molecular alterations of resistant cells. Cancer cell resistance to cisplatin has been described to be multifactorial, with several mechanisms operating simultaneously in a single cell [1,2]. Until now, reduced drug accumulation, increased drug inactivation within the cell, enhanced DNA repair and increased DNA damage tolerance have been found to contribute to cisplatin resistance [3]. Nevertheless, the most common feature of the cells with acquired resistance to cisplatin is impaired cellular accumulation of the drug [4].

Despite much effort, the mechanisms cisplatin employs to enter tumour cells remain largely obscure. Recent findings point out at the involvement of copper transporter 1 (CTR1) in the uptake of the platinum drug [5,6]. The transporter appears to be clinically relevant, since high expression of CTR1 in patients with ovarian carcinoma was associated with good therapeutic response, while low levels of the protein lead to poor therapeutic outcome [7]. Deletion of CTR1 was reported to decrease cisplatin accumulation and to increase resistance in vitro and in vivo [8,9]. Transfection of human ovarian carcinoma cells with CTR1 led to enhanced cellular accumulation of the drug but failed to increase DNA platination and cell sensitivity to cisplatin [10]. Some studies demonstrated that exposure of human tumour cells to clinically relevant concentrations of cisplatin triggers rapid degradation and loss of CTR1 [11,12]. However, conflicting results showed that transfecting cervix carcinoma cells with the transporter did not lead to any changes in cellular accumulation and cytotoxicity of cisplatin [13]. Furthermore, expression of CTR1 in various cisplatin-resistant cell lines was reported to be similar to that in the respective sensitive counterparts [13–15].

Recent work from our laboratory presented characterisation of the cisplatin-sensitive/resistant human ovarian carcinoma cell line pair

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A2780/A2780cis regarding cellular platinum accumulation and efflux, cytotoxicity and expression of CTR1. Cellular platinum accumulation was significantly reduced in cisplatin-resistant cells as compared to their sensitive counterparts [16]. As no differences in cisplatin efflux between A2780 and A2780cis cells were observed, we concluded that primarily a decrease in cisplatin influx accounted for lower intracellular platinum concentrations in the resistant cell line. The A2780cis cell line exhibited 4.4-fold resistance to cisplatin, which correlated well with the decrease in the degree of DNA platination. Cisplatin-resistant cells expressed 1.5–1.8-fold lower levels of CTR1 suggesting a relationship between CTR1 expression, cisplatin uptake, DNA platination and cell sensitivity to the drug [16]. Interestingly, no reduction in CTR1 expression levels after cisplatin exposure was observed in both cell lines [16]. The present study aimed at further clarification of the role of CTR1 as a determinant of cisplatin uptake and resistance to the drug in the A2780/A2780cis cell line pair.

This paper describes a detailed investigation of sub cellular localisation of CTR1 under various conditions including exposure to copper and cisplatin, copper influence on cellular platinum accumulation and cytotoxicity, as well as studies of co-localisation between CTR1 and a cisplatin analogue labelled with fluorogenic carboxyfluorescein-diacetate. Based on the results of the study, relevance of CTR1 for cisplatin accumulation and drug resistance in the above-mentioned cell line pair is discussed.

2. Experimental

2.1. Materials

Methyl-β-cyclodextrin, MTT (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide), Triton X-100, bovine serum albumin (BSA), ribonuclease A (RNAse A), GelMount™ mounting medium and *cis*-diamminedichloridoplatinum(II) (cisplatin) were obtained from Sigma (Steinheim, Germany). Copper(II) sulphate pentahydrate and Tween 20 were ordered from AppliChem (Darmstadt, Germany), bathocuproine disulfonic acid (BCS) was received from Acros (Geel, Belgium), and concentrated nitric acid (65%, v/v) from Merck (Darmstadt, Germany). ALEXA Fluor™ 488- and ALEXA Fluor™ 594-conjugated chicken anti-goat antibodies, DAPI (4′,6-diamidino-2-phenylindole), propidium iodide, ALEXA Fluor™ 594-conjugated chicken anti-rabbit antibody and NBD-C_6-ceramide (6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)-sphingosine) complexed to BSA were ordered form Invitrogen (Karlsruhe, Germany). Goat antibody to CTR1 (raised against a peptide mapping within an internal region of hCTR1), rabbit antibody to CTR1 (raised against amino acids 1–190 representing full length hCTR1) and horseradish peroxidase-conjugated anti-rabbit antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated anti-mouse antibody was ordered from Calbiochem, Merck KGaA, Darmstadt, Germany. [{1-([5-(and-6)-carboxyfluorescein-diacetate]aminomethyl)-1,2-ethylenediamine}-dichlorideplatinum(II)] (CFDA-Pt) was synthesised according to the previously published method [17].

2.2. Cell lines and growth conditions

The A2780 and A2780cis (cisplatin-resistant) human ovarian carcinoma cell lines were obtained from the European Collection of Cell Cultures, United Kingdom. Cell backups were frozen with 10% dimethylsulfoxide. The cell lines were grown as monolayers in RPMI 1640 medium supplemented with 10% foetal calf serum, 0.6 mM L-glutamine, 100 I.E./mL penicillin and 0.1 mg/mL streptomycin (Sigma, Steinheim, Germany) in a humidified atmosphere containing 5% CO_2. Every 10 passages a new backup of cells was thawed to maintain the resistance mechanisms unchanged during the cultivation.

2.3. Evaluation of cytotoxicity

Tumour cell sensitivity to copper sulphate and cisplatin in A2780 and A2780cis cells was evaluated using an MTT-based assay [18]. After trypsinisation, the cells were divided in 96-well plates at concentrations of 10^4 cells per well in 100 μL growth medium. The cells were allowed to attach overnight. Then, medium was removed and stock solutions of cisplatin (2 mg/mL) or copper sulphate (10 mM) in millipore water were diluted in medium. In order to assess copper influence on the cytotoxicity of cisplatin, copper sulphate was added to the cisplatin dilutions to a final concentration of 20 μM or 50 μM. Using different copper concentrations enables observing whether the effect of copper on cisplatin cytotoxicity is concentration-dependent. Six different dilutions each were added to the cells in quadruplicate (100 μL per well). After 72 h of incubation, 50 μL of 5 mg/mL MTT solution in phosphate buffered saline (PBS) was added to each well, and the cells were incubated at 37 °C for about 90 min. Subsequently, medium was discarded and 100 μL of dimethylsulfoxide was added to each well, yielding purple solutions. The optical density was measured at 590 nm using a Multiskan™ microplate reader (ThermoLabsystems, Dreieich, Germany). The results were analysed and the pEC_{50} values ($pEC_{50} = -\log EC_{50}$, EC_{50} is the drug concentration that produces 50% of the maximum possible response) were determined with the GraphPad Prism™ analysis software package (GraphPad Software, San Diego, USA) using non-linear regression (sigmoidal dose response, variable slope).

2.4. Cellular platinum accumulation

For the characterisation of cellular platinum accumulation, approximately 2×10^6 cells were incubated with 100 μM cisplatin or 100 μM CFDA-Pt in the culture medium up to 1 h. In order to investigate platinum accumulation in the presence of copper, copper sulphate was added to the cisplatin-containing medium to a final concentration of 50 μM or 200 μM. Using different copper concentrations allows observing whether the effect of copper on cisplatin accumulation is concentration-dependent. In some experiments, before cisplatin addition the cells were allowed to grow for 48 h in the medium containing 50 μM BCS. After certain time points the medium was discarded quickly and cells were washed with 1 mL ice-cold PBS (phosphate buffered saline, pH adjusted to 7.4). Then cells were trypsinised, resuspended in fresh drug-free medium and centrifuged for 1 min at 4 °C and 1520 *g*. The supernatant was discarded and the pellet was washed twice in 1 mL ice-cold PBS. After centrifugation for 1 min at 18,620 g the supernatant was discarded again and the cell pellet was frozen at −20 °C until further analysis. Immediately after thawing the cells were lysed in concentrated nitric acid for 1 h on a water bath at 80 °C. The cell pellets could be lysed completely yielding clear solutions. Then intracellular platinum concentrations were measured by flameless atomic absorption spectrometry (FAAS) using a validated modification of the procedure described by Kloft et al. [19] In brief, an atomic absorption spectrometer (SpectrAA™ Zeeman 220; Varian, Darmstadt, Germany) equipped with a graphite tube atomizer (GTA 100), a programmable sample dispenser (PSD 100) and a platinum hollow cathode lamp (UltrAA™ lamp) were used. The temperature programme included an ashing step at 1300 °C and an atomization step at 2700 °C. The lower limit of quantification (LLOQ) was 1 ng/mL. Platinum concentrations were calculated in relation to the number of cells (as measured with Casy™1 cell counter, Schärfe System, Reutlingen, Germany).

2.5. Immunohistochemical staining

In general, immunohistochemical staining was done one day after seeding cells on cover slips. In some experiments, cells were allowed

to grow on cover slips for 48 h in the presence of 50 μM BCS. Subsequently, some cells were treated with either cisplatin (0.5 μM, 2 μM or 5 μM), CFDA-Pt (5 μM), methyl-β-cyclodextrin (10 mM) or copper sulphate (100 μM or 500 μM) for different periods of time. Different concentrations of cisplatin and copper sulphate were used in order to investigate whether the concentration has an impact on sub cellular localisation of CTR1. After that, in some experiments the cells were further incubated with a drug-free medium for 1 h. Treatment of the cells with various substances was carried out at 37 °C unless otherwise stated. After three rinses with PBS, cells were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. After fixation, cells were washed three times with PBS and permeabilised with 0.5% Triton X-100 in PBS for 30 min. In parallel experiments the cells were permeabilised with 0.02% Triton X-100 in PBS for 30 min. No differences in the staining results depending on Triton X-100 concentration were observed. Labelling of the trans-Golgi network was performed with 5 μM NBD-C_6-ceramide complexed to BSA at 37 °C for 30 min. Cells were then rinsed with PBS, blocked for 1 h in PBS containing 1% BSA followed by incubation for 90 min at 37 °C with a primary antibody against CTR1 (diluted 1:10). A goat antibody to CTR1 raised against a peptide mapping within an internal region of hCTR1 was used unless otherwise stated. Subsequently, cells were washed with PBS and incubated for 90 min at 37 °C with an appropriate secondary ALEXA Fluor™ 488- or ALEXA Fluor™ 594-conjugated antibody (diluted 1:100). Antibody solutions were diluted in PBS containing 1% BSA. To stain the nucleus with propidium iodide in some samples, cells were treated with RNAse A (100 μg/mL in PBS) for 30 min at 37 °C, washed with PBS and incubated with 5 μM propidium iodide in PBS for 15 min at 37 °C. In some experiments, nuclei were stained with DAPI at the final concentration of 5 μg/mL. After the final washing steps, the cells were gradually dehydrated in an ethanol series of 70%, 90%, 100%, for 1 min each. The cover slips were mounted in GelMount™ medium for microscopic observations. Images are each representative of six to eight images taken using a Leica TCS SP2 confocal system at Laboratory for Molecular Developmental Biology, LIMES Institute, University of Bonn or using a Nikon A1 confocal system at the Institute of Pharmacy, University of Bonn. Each fluorochrome was scanned individually. Distribution of CTR1 away from the nucleus was determined manually as a maximum distance between the nucleus fluorescence and the protein immunofluorescence measured along the distribution radius.

2.6. Western blot analysis

Cells were grown in T175 flasks until they reached 80%–90% confluence and then exposed to 2 μM cisplatin, 100 μM copper sulphate, or medium alone for 5 min. After exposure, cells were briefly washed with cold PBS without Ca^{2+} and Mg^{2+}. Then 1 mL lysis buffer (20 mM Tris, 137 mM NaCl, 10% glycerine, 1% tergitol NP-40, 2 mM EDTA, 1 mM Na_3VO_4 at (pH 10), 1 μM leupeptin, 2 μM pepstatin A) per flask was added and the cells were collected using cell scrapper. The cells were lysed on ice for 30 min. Subsequently, the suspensions were centrifuged for 5 min at 18,620 *g* at 4 °C, and the protein contents in the supernatants were measured using bicinchonic acid assay (BCA™ Protein Assay Kit, Pierce, Rockford, IL, USA). The samples containing 40 μg protein were heated to 95 °C for 5 min before electrophoresis in 10% SDS-polyacrylamide electrophoresis gel. Transfer to polyvinylidene fluoride membrane (Carl Roth GmbH, Karlsruhe, Germany) was performed electrophoretically for 60 min at 100 V using a Mini-Protean II apparatus (Bio-Rad Laboratories GmbH, Munich, Germany). The membrane was blocked in 5% BSA in Tris-buffered saline containing 0.2% Tween 20 for 1 h at room temperature. Blot was incubated at 4 °C overnight with the antibody against human CTR1 diluted 1:75 in Tris-buffered saline containing 0.2% Tween 20 and 5% BSA. Subsequently, the membrane was washed three times with Tris-buffered saline containing 0.2% Tween 20 and incubated for 1 h at room temperature with the horseradish peroxidase-conjugated anti-rabbit antibody diluted 1:1000 in Tris-buffered saline containing 0.2% Tween 20 and 5% BSA. Before detection of human CTR1, the membrane was again washed three times with Tris-buffered saline containing 0.2% Tween 20, and the detection was performed using a Molecular Imager ChemiDoc™ XRS + System from Bio-Rad Laboratories GmbH, Munich, Germany, according to manufacturer's instructions. Then the membrane was washed three times with Tris-buffered saline containing 0.2% Tween 20 and incubated for 1 h at room temperature with the antibody against β-actin diluted 1:200 in Tris-buffered saline containing 0.2% Tween 20 and 5% BSA. Subsequently, the membrane was washed again three times with Tris-buffered saline containing 0.2% Tween-20 and incubated for 1 h at room temperature with the horseradish peroxidase-conjugated anti-mouse antibody diluted 1:1000 in Tris-buffered saline containing 0.2% Tween 20 and 5% milk powder. Before detection of β-actin, the membrane was again washed three times with Tris-buffered saline containing 0.2% Tween 20, and the detection was performed using the above-mentioned detection system. Densitometric analysis was performed using ImageLab™ software.

2.7. Statistics

The significance of differences was analysed using Student's *t* test unless otherwise stated. p Values of <0.05 were considered significant.

3. Results

3.1. Effect of copper on cellular platinum accumulation and cisplatin cytotoxicity

Given a diminished cisplatin accumulation and a decreased level of CTR1 in cisplatin-resistant A2780cis cells as compared to their sensitive counterparts [16], we assumed that CTR1 is involved in the uptake of the drug in A2780/A2780cis cell line pair. The initial experiments to confirm this hypothesis were the studies of the copper influence on cisplatin accumulation and cytotoxicity in the sensitive and resistant cell lines.

First, the sensitivity of A2780 and A2780cis cells to cisplatin and to copper sulphate alone was assessed. The EC_{50} values for cisplatin in A2780 and in A2780cis were determined as 2.9 μM ($pEC_{50} = 5.54 \pm 0.03$) and 14.9 μM ($pEC_{50} = 4.83 \pm 0.04$), respectively, and for copper sulphate — as 132 μM ($pEC_{50} = 3.93 \pm 0.01$) and 362 μM ($pEC_{50} = 3.42 \pm 0.03$), respectively (pEC_{50} data represent mean values ± SEM of five experiments). As expected, the A2780cis cell line was found resistant to cisplatin and to a lesser extent to copper (resistant factors were calculated as 5.1 for cisplatin and 2.7 for copper). This is in agreement with the previous findings that cisplatin-resistant cells often exhibit cross-resistance to copper and that in these cells the degree of cisplatin resistance is higher [20].

Subsequently, the influence of co-incubation with copper(II) sulphate on cellular accumulation of cisplatin in both cell lines was studied. CTR1 is known to be Cu(I)-specific. Extracellular copper exists in its oxidised form, however, it is reduced from Cu(II) to Cu(I) by the membrane-bound cupric reductases before being taken up [21]. As Fig. 1 shows, cisplatin accumulation after simultaneous incubation with copper sulphate at two different concentrations was not significantly different from that after treatment with cisplatin alone. This indicates that cisplatin and copper are not likely to compete for the uptake via CTR1 in our sensitive/resistant cell line pair. It may be argued that high cisplatin concentration used in the experiment (100 μM) could lead to cross-linking of amino acid residues of CTR1 thereby impairing its transport function. However, the data from Guo et al. showed that treatment with 200 μM cisplatin did not inhibit copper-stimulated endocytosis of CTR1 [22]. Low concentrations of cisplatin (0.5 μM and 2 μM) have been reported to

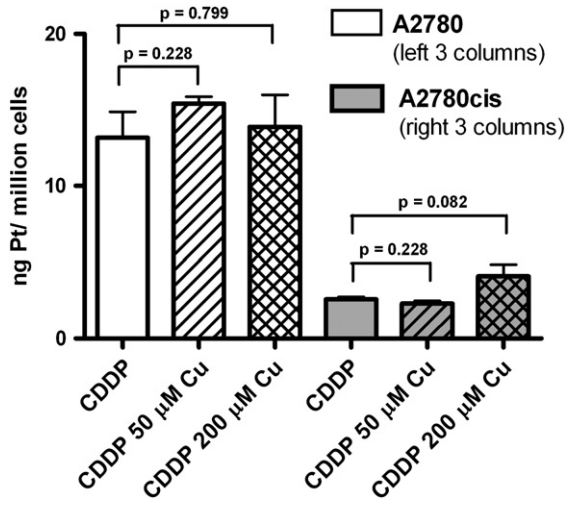

Fig. 1. Effect of copper on cellular accumulation of cisplatin. Cellular platinum accumulation [ng Pt/million cells, n = 6, mean ± SEM] in A2780 and A2780cis cells after 1 h incubation with 100 μM cisplatin alone, with 100 μM cisplatin and 50 μM copper sulphate or with 100 μM cisplatin and 200 μM copper sulphate simultaneously.

downregulate the copper transporter, however, that was not the case at higher concentrations (10 μM and 200 μM) [12].

Further, the effect of different copper concentrations on the cytotoxicity of cisplatin was assessed in both A2780 and A2780cis. Lower concentrations of copper in comparison with the cellular accumulation experiments were chosen in order to exclude the toxic effect of copper sulphate alone on the cells upon incubation over 72 h. Tumour cell sensitivity to cisplatin was not affected by the presence of copper in culture medium (Table 1). These results are in agreement with no influence of copper on cisplatin accumulation.

3.2. Sub cellular localisation of CTR1

Since altered sub cellular localisation of transport proteins has been earlier suggested to have an impact on their function [23,24], localisation of CTR1 in A2780 and A2780cis cells was investigated. As shown in Fig. 2A, CTR1 was mainly localised in the perinuclear region. This result was further confirmed using another antibody against CTR1 (Fig. 2B). As is clear from the co-localisation experiments with a fluorescent marker for the trans-Golgi network, CTR1 was partly localised in the trans-Golgi in both cell lines (Fig. 2C). Localisation of the copper transporter in the trans-Golgi network has previously been reported in other cell line models [25]. It has been suggested that CTR1 steadily recycles between the trans-Golgi and the cell membrane, where it mediates copper uptake. The protein has been proposed to localise in the trans-Golgi network by a process of continuous retrieval from the cell surface [25]. In order to reveal whether CTR1 cycles through different compartments of our cells, A2780 and A2780cis cells were cultured in the presence of methyl-β-cyclodextrin, which is known to bind to cholesterol in the membranes and to inhibit clathrin- and caveolae-mediated endocytosis [26]. In both sensitive and resistant cells pre-treated with methyl-β-cyclodextrin, CTR1 was no longer localised in the perinuclear region, but redistributed to vesicular structures diffusely located throughout the cell (Fig. 2D). Quantitative analysis showed that CTR1 was distributed significantly further away from nucleus after incubation of the cells with methyl-β-cyclodextrin (Table 2). Thus, our results indicate that a dynamic process, which involves endocytosis from the cell surface, determines, at least in part, the steady-state localisation of CTR1 in the perinuclear region.

In the next step, the influence of cisplatin and copper sulphate on sub cellular localisation of CTR1 was studied. In sensitive, as well as in resistant cell lines, copper treatment both at 100 μM and 500 μM resulted in redistribution of CTR1 to vesicles dispersed throughout the cytoplasm. Incubation with different copper concentrations led to similar results. Interestingly, protein localisation in the perinuclear region was restored 1 h after copper withdrawal from the culture medium (Fig. 3A). Median distribution of CTR1 away from the nucleus was found significantly higher after treatment of the cells with copper sulphate (Table 2). Similarly, after removal of copper from the culture medium the transporter was localised significantly less far away from the nucleus than in the presence of copper (Table 2). These results suggest that CTR1 is functional with regard to copper uptake in A2780 and A2780cis cells.

In contrast, incubation with cisplatin at the concentrations of 0.5 μM, 2 μM or 5 μM had no remarkable effect on localisation of the CTR1 within both A2780 and A2780cis cells (Fig. 3B). Also in this case, the results were similarly independent on cisplatin concentration used in the experiments. After treatment with cisplatin, CTR1 was located only slightly closer to the nucleus than in untreated cells. This result was slightly significant only in the sensitive cells. In addition, no remarkable reduction in CTR1 staining as reported previously [11,12] was observed in any case.

3.3. Influence of copper chelation on CTR1

Some previous reports on the sub cellular localisation of CTR1 in A2780 cells described both membrane and intracellular localisations of the protein in this cell line [10,11]. However, in these earlier studies the cells were cultured in the presence of a copper chelating agent, bathocuproine disulfonic acid, before staining. In order to investigate the influence of copper deprivation from culture medium on CTR1 and in attempt to resolve the discrepancy between our results and the literature data, we studied the sub cellular localisation of CTR1 in A2780 and A2780cis cells after prolonged treatment with BCS. The images presented in Fig. 4A show that the conditions of copper shortage caused redistribution of the transporter to the plasma membrane and to vesicular structures diffusely located throughout the cell. Interestingly, membrane localisation of CTR1 was not affected by cisplatin exposure regardless of the drug concentration used (Fig. 4A). Incubation of the BCS-pretreated cells with copper sulphate both at 100 μM or 500 μM resulted in redistribution of CTR1

Table 1
Sensitivity of A2780 and A2780cis cells [pEC_{50}, n = 5, mean ± SEM] to cisplatin and to cisplatin in the presence of 20 μM or 50 μM copper sulphate.

	CDDP		CDDP + 20 μM $CuSO_4$		CDDP + 50 μM $CuSO_4$	
	pEC_{50} (EC_{50}, μM)	p	pEC_{50} (EC_{50}, μM)	p*	pEC_{50} (EC_{50}, μM)	p*
A2780	5.54 ± 0.03 (2.88)	n.a.	5.50 ± 0.04 (3.15)	0.489	5.50 ± 0.1 (3.10)	0.783
A2780cis	4.83 ± 0.04 (14.89)	n.a.	4.89 ± 0.02 (12.76)	0.15	4.89 ± 0.02 (12.94)	0.20

n.a. = not applicable.
* Difference in comparison to the respective cells treated with cisplatin alone.

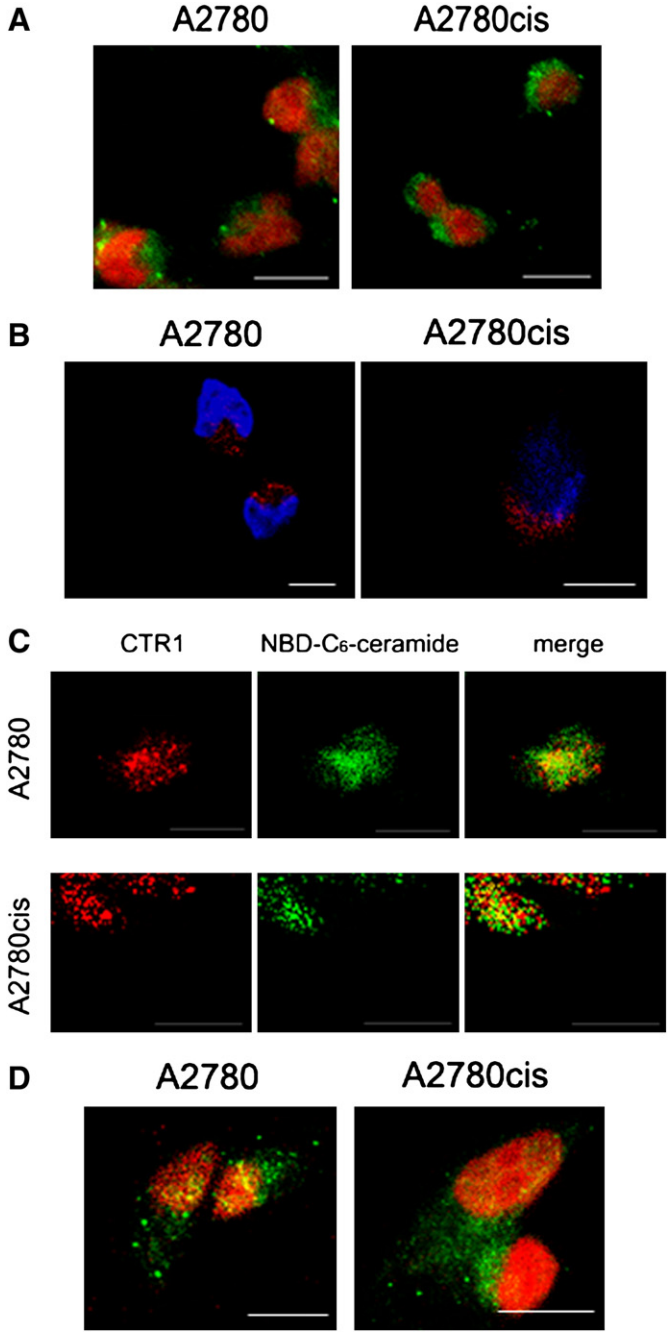

Fig. 2. Sub cellular localisation of CTR1 in A2780 and A2780cis cells. A. Immunofluorescence localisation of CTR1 (*green*) in A2780 and A2780cis cells. CTR1 is detected using a goat antibody raised against a peptide mapping within an internal region of hCTR1. Cell nuclei were stained with propidium iodide (*red*). Scale bar, 10 μm. B. Immunofluorescence localisation of CTR1 (*red*) in A2780 and A2780cis cells. CTR1 is detected using a rabbit antibody raised against amino acids 1–190 representing full length hCTR1. Cell nuclei were stained with DAPI (*blue*). Scale bar, 10 μm. C. Co-localisation of the marker for CTR1 (*red*) with NBD-C_6-ceramide (*green*), a marker for the trans-Golgi network. *Yellow*, the structure is positive for Golgi and the protein marker. Scale bar, 10 μm. D. Immunofluorescence localisation of CTR1 (*green*) in A2780 and A2780cis cells cultured in the presence of 10 mM methyl-β-cyclodextrin in RPMI 1640 medium supplemented with 25 mM Hepes and 0.1% BSA for 1 h. Cell nuclei were stained with propidium iodide (*red*). Scale bar, 10 μm.

from the plasma membrane to the vesicles scattered throughout the cytoplasm (Fig. 4A).

Although cisplatin had no effect on membrane localisation of CTR1 in BCS-treated cells, involvement of the membrane CTR1 in cisplatin influx could not be ruled out. Thus, we investigated whether the presence of significant fraction of the transporter on the membrane facilitates cisplatin uptake. However, measurements of cellular platinum accumulation in BCS-treated and untreated cells showed that copper deprivation and the resulting re-localisation of CTR1 had no impact on cisplatin uptake (Fig. 4B).

Table 2
Median distribution of CTR1 away from the nucleus (μm, n = 35–76) in the untreated cells (−), cells treated with methyl-β-cyclodextrin for 1 h (+MCD), cells incubated with $CuSO_4$ for 1 h (+$CuSO_4$), cells treated with $CuSO_4$ for 1 h and subsequently with medium for 1 h (+$CuSO_4$, +medium) and cells incubated with cisplatin for 1 h (+CDDP). Significance of differences was analysed using Mann–Whitney *U* test in comparison to untreated cells unless otherwise stated.

	−	+MCD	+$CuSO_4$	+$CuSO_4$, +medium	+CDDP
A2780	1.930	4.780	3.450	1.900	1.650
A2780cis	2.140	4.080	2.915	1.535	1.860
p (A2780)	n.a.	<0.0001	<0.0001	<0.0001 *	0.017
p (A2780cis)	n.a.	<0.0001	<0.0001	<0.0001 *	0.074

n.a. = not applicable.
* Difference in comparison to cells treated with $CuSO_4$ alone.

3.4. Western blot analysis

According to earlier data from our laboratory, CTR1 gene expression in the resistant cell line is significantly reduced compared to the sensitive counterpart and cisplatin exposure does not influence expression levels as determined by quantitative real-time PCR [16]. In order to confirm these findings, the levels of CTR1 protein in untreated cells, cells exposed to cisplatin and cells treated with copper sulphate were assessed using western blot analysis. As is clear from Fig. 5, CTR1 protein levels are approximately two-fold lower in A2780cis cells than in the sensitive counterparts, and the difference was statistically significant ($p = 0.003$). In A2780 cells exposure to copper or cisplatin does not influence protein expression ($p = 0.694$ and 0.916 for copper and cisplatin, respectively). CTR1 levels in A2780cis cells were slightly increased after incubation with copper or cisplatin, however, in both cases the result was not statistically significant ($p = 0.283$ and 0.309 for copper and cisplatin, respectively).

3.5. Co-localisation with a fluorescent cisplatin analogue

In order to find out whether CTR1 directly mediates cisplatin uptake, co-localisation of the protein with a fluorescent cisplatin analogue CFDA-Pt (Fig. 6A) was studied. Fluorescent platinum complexes, and amongst others CFDA-Pt, have been widely used for investigation of the cellular trafficking of platinum drugs [17,27–30]. Modification of a platinum complex with a fluorescent tag enables visualisation of the compound and thereby monitoring of the drug trafficking within the cells. However, a labelled compound will only represent a suitable model for the respective platinum drug if a fluorescent label induces no significant changes of the properties of a platinum complex. Previous studies in the A2780 sensitive/resistant cell line pair showed that A2780cis cells are cross-resistant towards cisplatin and CFDA-Pt. This indicates that the labelled complex is a good model compound to study cisplatin resistance in A2780cis cells, as CFDA-Pt is susceptible to the resistance mechanisms active in this cell line [23]. In order to ensure the suitability of CFDA-Pt for the investigation of cisplatin uptake, cellular accumulation of the labelled complex as compared to cisplatin was assessed in the sensitive and resistant cell lines. In both cell lines, cellular accumulation of CFDA-Pt was markedly higher than that of cisplatin. This is likely to result from high lipophilicity of the label, which enhances passive diffusion. Passive diffusion, however, is not a determinant of

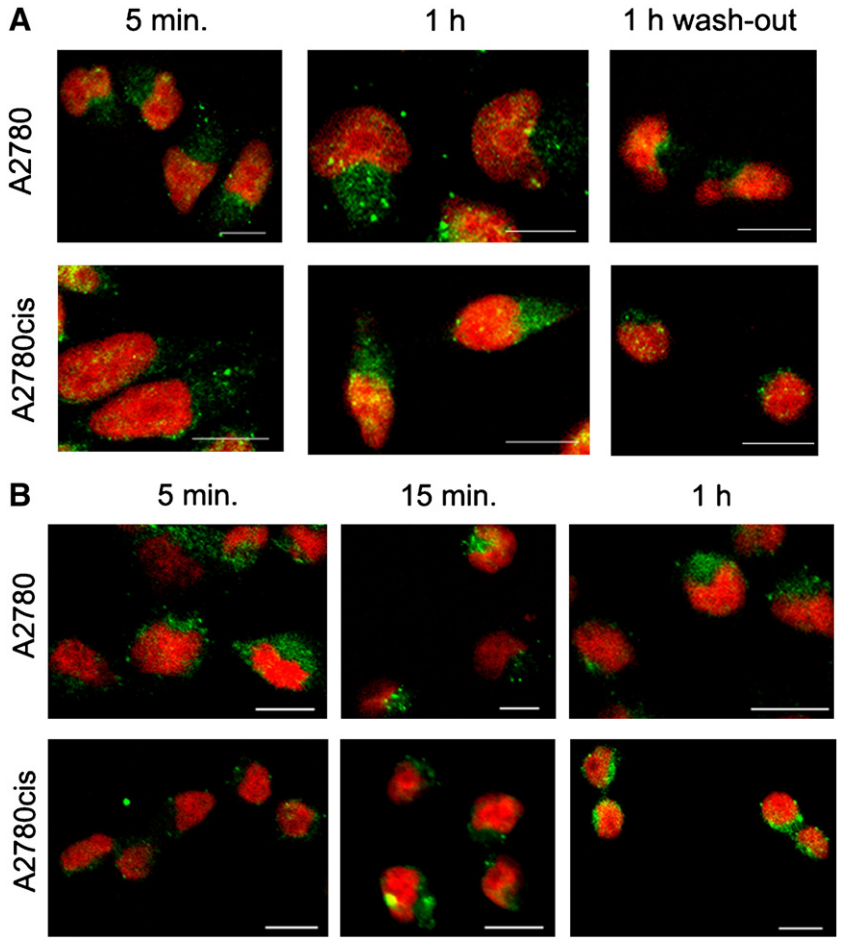

Fig. 3. Sub cellular localisation of CTR1 after exposure to cisplatin and copper sulphate. A. Immunofluorescence localisation of CTR1 (*green*) in A2780 and A2780cis cells after exposure to copper sulphate at 500 μM for 5 min, for 1 h and after subsequent incubation of the cells in the drug-free medium for 1 h. Cell nuclei were stained with propidium iodide (*red*). Scale bar, 10 μm. Similar images were obtained after incubation with 100 μM copper sulphate. B. Immunofluorescence localisation of CTR1 (*green*) in A2780 and A2780cis cells after exposure to 5 μM cisplatin for 5 min, for 15 min and for 1 h. Cell nuclei were stained with propidium iodide (*red*). Scale bar, 10 μm. Similar images were obtained after incubation with 0.5 μM and 2 μM cisplatin.

decreased cisplatin accumulation in resistant cells, which we attribute to the changes in the active transport system. The influence of the carboxyfluorescein-diacetate moiety on the active transport across the membrane appears unlikely, since accumulation of CFDA-Pt was significantly decreased in resistant A2780cis cells as compared to the sensitive A2780 cells, as was the case with cisplatin (Fig. 6B). These results present evidence that one of the important mechanisms of cisplatin resistance in A2780cis, reduced uptake, affects CFDA-Pt as well.

After 5 min, 15 min and 1 h incubation of A2780 and A2780cis cells with CFDA-Pt, no substantial co-localisation between the fluorescent cisplatin analogue and CTR1 was observed (Supplementary material, Fig. S1 and S2). On the other hand, interaction of cisplatin with CTR1 could be only transient and it is possible that given rapid recycling of the transporter between the intracellular compartment and the cell membrane co-localisation could not be detected. For that reason, co-localisation between CFDA-Pt and CTR1 was investigated upon inhibition of CTR1 continuous endocytosis from cell surface. The co-localisation was much stronger when methyl-β-cyclodextrin, which interferes with clathrin- and caveolae-mediated endocytosis [26], was added immediately after CFDA-Pt (Fig. 7A). If methyl-β-cyclodextrin was added before CFDA-Pt, some positive co-localisation could be observed but it was much less pronounced (Fig. 7B and C). This difference could be expected as after endocytosis inhibition CTR1 continuous recycling between the perinuclear region and the plasma membrane is much slower, and much less platinum can be taken up by CTR1. In another experiment, CFDA-Pt was added to the cells immediately after putting the cells on ice, which were further incubated with the complex at 4 °C. Clear co-localisation between CFDA-Pt and CTR1 was observed in both cell lines (Fig. 8). In all cases after inhibition of its continuous endocytosis from cell surface, copper transporter 1 exhibited diffused vesicular localisation, and positive co-localisation between CFDA-Pt and CTR1 was detected in vesicular structures (Figs. 7 and 8). It could be argued that much of CFDA-Pt probably enters the cells by passive diffusion and that this fraction co-localises with the transporter. However, compounds, which readily diffuse through the membrane(s), are unlikely to specifically accumulate in endocytic vesicles. Given that co-localisation was observed in vesicular structures, we believe that the fraction of CFDA-Pt taken up through passive diffusion does not co-localise with CTR1 to any significant extent. In addition, diffused vesicular localisation of the transporter after treatment with methyl-β-cyclodextrin or after incubation at 4 °C supports our conclusion regarding continuous recycling of CTR1 between the perinuclear region and the plasma membrane.

4. Discussion

Cisplatin resistance in A2780cis cells used in this study has been attributed to the diminished drug uptake, elevated glutathione levels

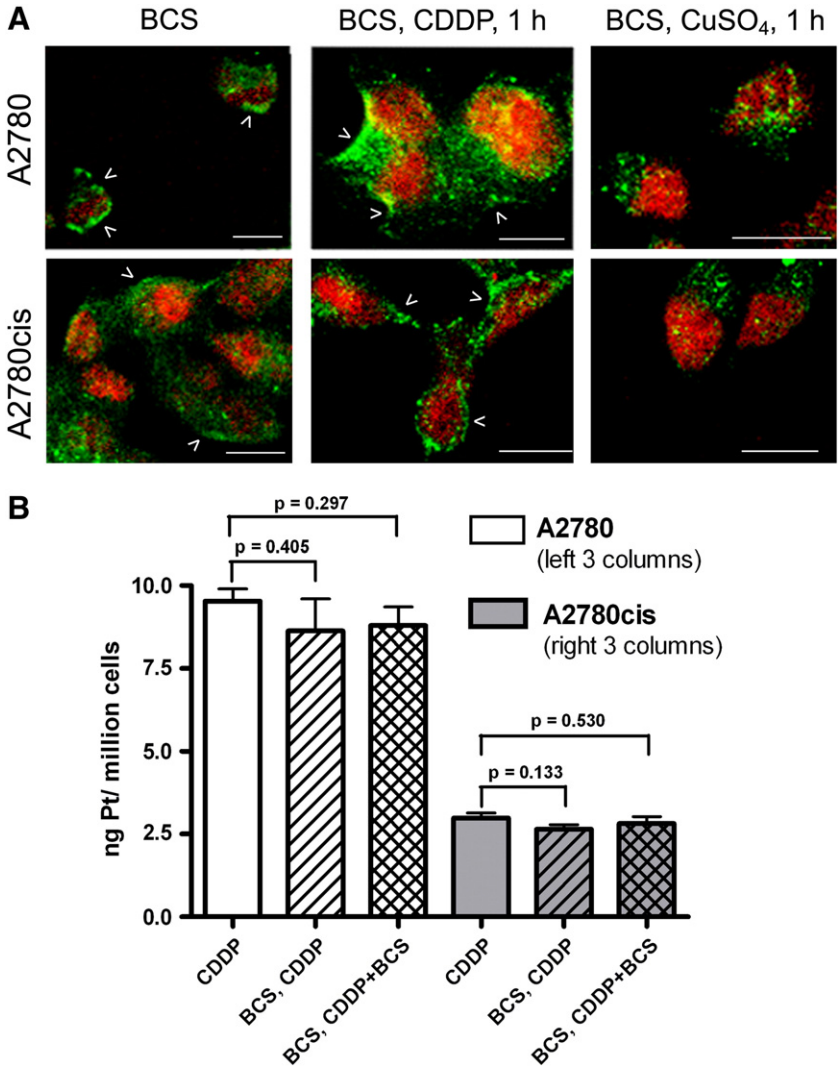

Fig. 4. Sub cellular localisation of CTR1 and cellular platinum accumulation under conditions of copper shortage. A. Immunofluorescence localisation of CTR1 (*green*) in A2780 and A2780cis cells cultured in the presence of 50 μM BCS for 48 h, after exposure of these cells to 5 μM cisplatin for 1 h and after incubation with 500 μM copper sulphate for 1 h. Cell nuclei were stained with propidium iodide (*red*). Open arrowheads indicate plasma membranes. Scale bar, 10 μm. Similar images were obtained after exposure to 100 μM copper sulphate or 0.5 μM and 2 μM cisplatin, respectively. B. Cellular platinum accumulation [ng Pt/million cells, n = 6, mean ± SEM] in A2780 and A2780cis cells after exposure to 100 μM cisplatin for 1 h, and in the cells cultured in the presence of 50 μM BCS for 48 h — after 1 h incubation with 100 μM cisplatin alone and with 100 μM cisplatin and 50 μM BCS simultaneously.

and intracellular sequestration of cisplatin [16,23]. CTR1 expression in the resistant cells is decreased compared to the sensitive counterparts on mRNA [16] and on protein level. These findings pointed out at the relevance of reduced CTR1 expression for diminished cisplatin uptake and for resistance to the platinum drug in the A2780cis cell line. In accordance with this hypothesis, cisplatin-resistant A2780cis cells were also found resistant to copper sulphate. However, the presence of copper in the culture medium had no impact on cisplatin accumulation and cytotoxicity in both cell lines. These results indicate that CTR1 may employ different mechanisms for cisplatin and copper transport across the plasma membrane as has been suggested earlier [31]. This would not be surprising given different coordination chemistries of Pt(II) and Cu(I).

In basal medium CTR1 was predominantly localised in the perinuclear region in both cell lines. An earlier report from Klomp et al. showed that cell surface localisation of CTR1 is cell-type specific [25]. In cells featuring intracellular localisation of the transporter, CTR1 was suggested to rapidly recycle between the intracellular compartment and the cell surface, where it mediates copper uptake [25]. In our cell line system as well, the steady-state localisation of copper transporter 1 in perinuclear region appears to involve continuous endocytosis of the protein from the cell membrane.

Earlier Holzer et al. reported a significant fraction of CTR1 to be localised on the plasma membrane in A2780 cells [10,11]. However, in their experiments the cells were cultured in the presence of a copper chelating agent. Under these conditions, CTR1 was present on the membrane in our cells as well. This result highlights the importance of the copper status in the culture medium on the sub cellular localisation of CTR1. According to one earlier study, a copper chelator BCS can also significantly influence CTR1 expression [21]. The RPMI 1640 medium used in our and in the earlier studies is known to be devoid of transition metals such as iron, copper, zinc and manganese [32]. A major source of copper in the culture medium is added serum [33]. Copper concentration in RPMI 1640 supplemented with 20% foetal calf serum was determined as 1.02 μM [34]. In our and previously reported [10,11] experiments, the culture medium contained 10% serum. Thus, copper content in this case can be estimated as approximately 0.5 μM. Apparently, even such small copper concentrations can affect sub cellular localisation of CTR1. Copper levels in human blood have been reported to range between 15 and 20 μM

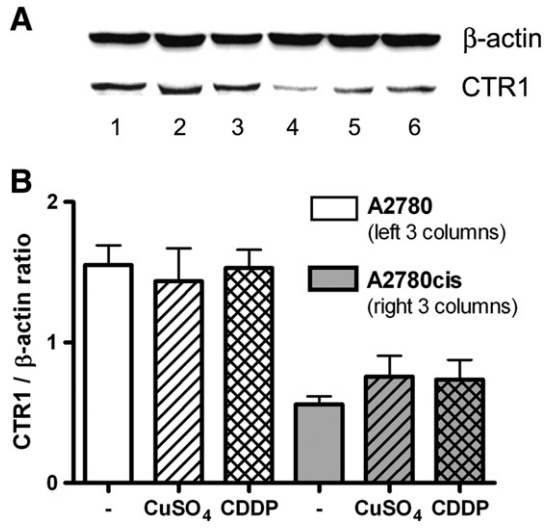

Fig. 5. Expression of CTR1 protein in A2780 and A2780cis cells. A. Representative Western blot analysis of CTR1 protein expression in untreated A2780 and A2780cis cells (lines 1 and 4, respectively), in A2780 and A2780cis cells exposed to 100 μM copper sulphate (lines 2 and 5, respectively) for 5 min, and in A2780 and A2780cis cells treated with 2 μM cisplatin (lines 3 and 6, respectively) for 5 min. B. Densitometric analysis of CTR1 protein expression related to the protein expression of β-actin (n = 3 ± SEM) in untreated cells (−), in cells exposed to 100 μM copper sulphate ($CuSO_4$) for 5 min, and in cells treated with 2 μM cisplatin (CDDP) for 5 min.

[35], and are therefore even higher than copper content in the culture medium. For this reason, experiments conducted in the absence of the copper chelating agents reflect the in vivo situation much better than those performed upon copper deficiency.

Regardless of the copper status in culture medium, cisplatin exposure had no remarkable effect on sub cellular localisation of CTR1 in both cell lines. In contrast, treatment with copper sulphate caused changes in CTR1 distribution within the cells. Different behaviours of the transporter upon cisplatin and copper exposure support the conclusion that CTR1 employs different mechanisms for their transport. Our observations that cisplatin treatment does not induce loss of CTR1 contradict previous findings of Holzer et al. regarding proteasomal degradation of CTR1 upon cisplatin exposure in A2780 and 2008 ovarian carcinoma cells [11,12]. However, our results were confirmed by the findings that in the cell lines studied cisplatin exposure has no impact on the CTR1 expression on mRNA [16] and protein level. Given exactly the same conditions used in our and previous [11,12] experiments, the discrepancy in the results obtained is puzzling. One feature distinguishing our cell lines from the ones used by Holzer and Howell may be different cellular levels of the metallochaperone Atox1. This metallochaperone has been shown to be essential for cisplatin-induced degradation of CTR1 [36].

The fact that cisplatin exposure does not affect localisation and expression of CTR1 does not rule out the involvement of CTR1 in cisplatin transport. For this reason, co-localisation of the transporter and the cisplatin analogue modified with carboxyfluorescein-diacetate was investigated. Fluorescent cisplatin derivatives have been widely used to study the involvement of other transporters (e.g. ATP7A, ATP7B, MRP2) in the intracellular trafficking of cisplatin [30,37]. To the best of our knowledge, this is the first report of co-localisation experiments between CTR1 and a fluorescent cisplatin analogue. In both A2780 and A2780cis cells, co-localisation between CFDA-Pt and CTR1 could only be detected upon inhibition of CTR1 continuous recycling between the intracellular compartment and cell surface. Co-localisation was observed in the sensitive as well as in the resistant cell lines suggesting that changes in CTR1 expression and not in its function account for reduced platinum accumulation in A2780cis cells. Interestingly, co-localisation was detected in vesicular structures. This finding implies that cisplatin may be quickly endocytosed together with CTR1 while being transported across the plasma membrane by the protein. This would explain why cisplatin had no influence on sub cellular localisation of CTR1. It remains unclear whether cisplatin is bound to CTR1 during internalisation or not. Also the nature of CTR1-expressing vesicles containing CFDA-Pt is still to be identified. Several earlier studies have suggested cisplatin accumulation in vesicles, probably heading to Golgi apparatus [29,30]. Safaei et al. have detected another fluorescent cisplatin analogue in recycling endosomes [30], which may be the case in our cell line pair as well given continuous recycling of CTR1 between the perinuclear region and cell membrane. However, exact intracellular fate of cisplatin-containing vesicles and how and where the drug is released remains to be determined.

In this work, we use co-localisation experiments for the first time in the literature to strengthen earlier conclusions that CTR1 mediates cisplatin uptake. The protein appears to transport the platinum drug not only in sensitive, but also in cisplatin-resistant cell lines. This indicates that reduced cisplatin accumulation, a well-documented

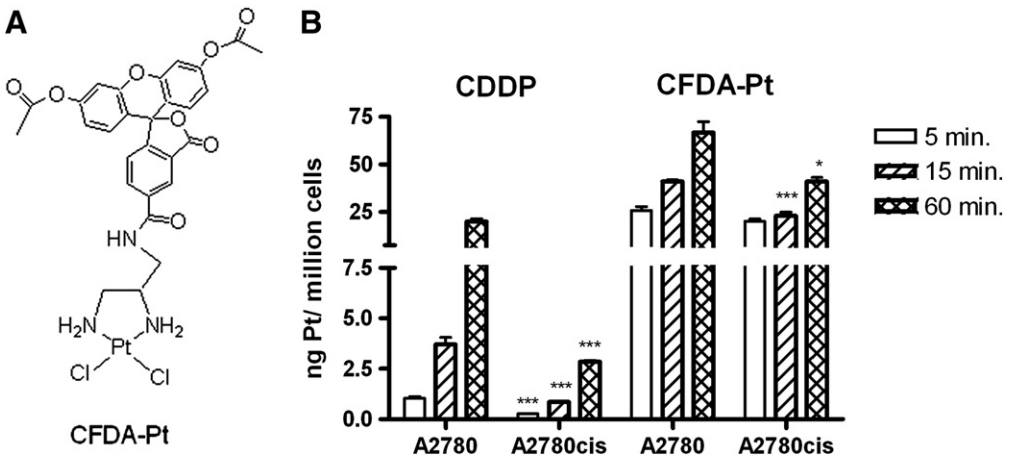

Fig. 6. Cellular accumulation of CFDA-Pt. A. Chemical structure of the fluorescent cisplatin analogue (CFDA-Pt). B. Cellular platinum accumulation [ng Pt/million cells, n = 3–6, mean ± SEM] in A2780 and A2780cis cells after exposure to 100 μM cisplatin and to 100 μM CFDA-Pt for 5 min, 15 min and 1 h. For the analysis of significance of differences, cellular platinum accumulation in A2780cis cells was compared to that in A2780 cells after the same period of incubation with cisplatin or CFDA-Pt. * $p<0.05$, *** $p<0.001$.

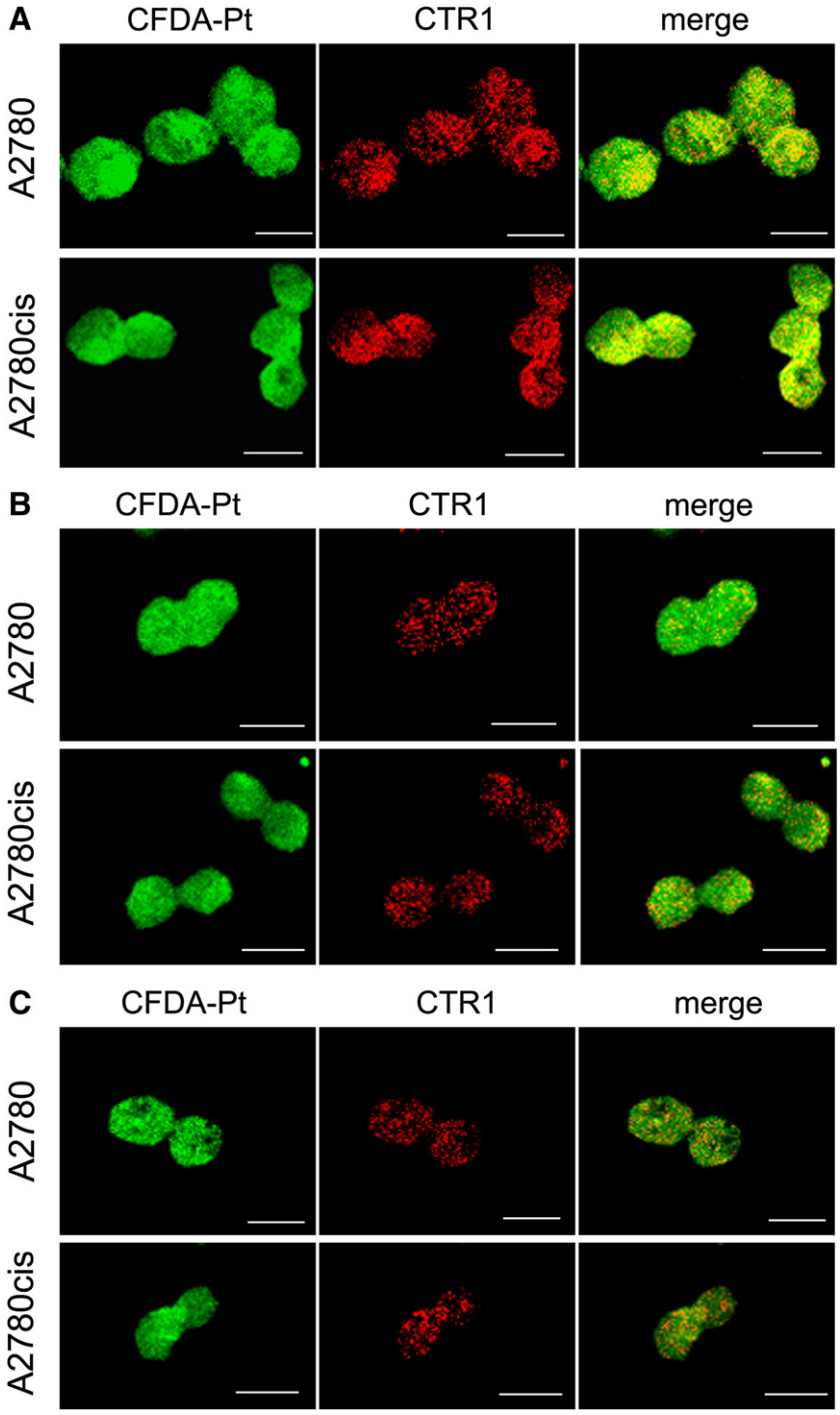

Fig. 7. Co-localisation of CFDA-Pt and the marker for CTR1 upon endocytosis inhibition with methyl-β-cyclodextrin. A. Co-localisation of the marker for CTR1 (*red*) and CFDA-Pt (*green*) after simultaneous incubation of A2780 and A2780cis cells with 5 μM CFDA-Pt and 10 mM methyl-β-cyclodextrin in RPMI 1640 medium supplemented with 25 mM Hepes and 0.1% BSA for 30 min. Methyl-β-cyclodextrin was added to the cells immediately after CFDA-Pt. *Yellow*, the structure is positive for CFDA-Pt and the protein marker. Scale bar, 10 μm. B. Co-localisation of the marker for CTR1 (*red*) and CFDA-Pt (*green*) after pre-incubation of A2780 and A2780cis cells with 10 mM methyl-β-cyclodextrin in RPMI 1640 medium supplemented with 25 mM Hepes and 0.1% BSA for 30 min and subsequent incubation with 5 μM CFDA-Pt in full growth medium for 30 min. *Yellow*, the structure is positive for CFDA-Pt and the protein marker. Scale bar, 10 μm. C. Co-localisation of the marker for CTR1 (*red*) and CFDA-Pt (*green*) after pre-incubation of A2780 and A2780cis cells with 10 mM methyl-β-cyclodextrin in RPMI 1640 medium supplemented with 25 mM Hepes and 0.1% BSA for 30 min and subsequent co-incubation with methyl-β-cyclodextrin and 5 μM CFDA-Pt for 30 min. *Yellow*, the structure is positive for CFDA-Pt and the protein marker. Scale bar, 10 μm.

mechanism of platinum drug resistance in A2780cis cells, can be attributed to the decreased CTR1 expression and not to its impaired function. We also suggest for the first time that being transported across the membrane by CTR1 cisplatin is likely to be internalised together with the protein as a part of CTR1 continuous recycling between the intracellular compartment and cell surface.

Abbreviations

CTR1	copper transporter 1
BSA	bovine serum albumin
BCS	bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) disulfonic acid
PBS	phosphate buffered saline

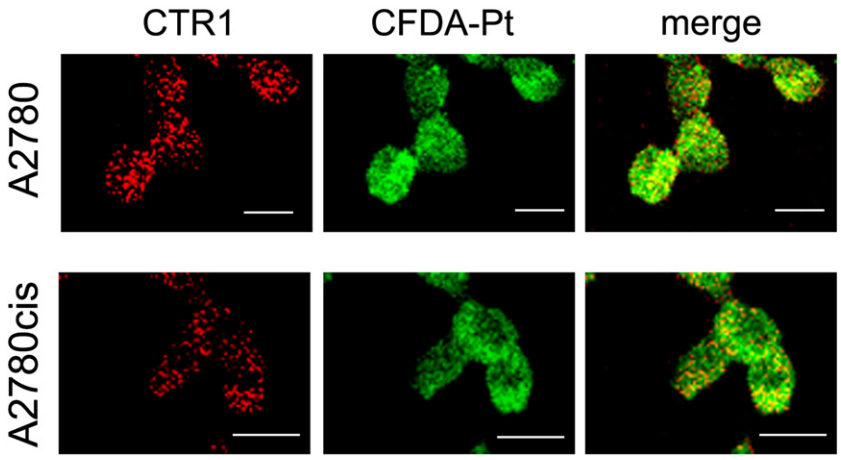

Fig. 8. Co-localisation of CFDA-Pt and the marker for CTR1 at 4 °C. Co-localisation of the marker for CTR1 (*red*) and CFDA-Pt (*green*) after 5 min incubation of A2780 and A2780cis cells with 5 μM CFDA-Pt at 4 °C. CFDA-Pt was added to the cells immediately after putting them on ice followed by incubation at 4 °C. *Yellow*, the structure is positive for CFDA-Pt and the protein marker. Scale bar, 10 μm.

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2 H-tetrazolium bromide
DAPI	4′,6-diamidino-2-phenylindole
ATP7A	ATPase, copper transporting, alfa polypeptide
ATP7B	ATPase, copper transporting, beta polypeptide
MRP2	multidrug resistance protein 2
CDDP	*cis*-diamminedichloroplatinum (II), cisplatin.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jinorgbio.2012.07.010.

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